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ORIGINAL ARTICLE

Mansoa alliacea extract improves the growth performance and innate immune response of *Arapaima gigas* challenged with *Aeromonas hydrophila* and handling stress

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ABSTRACT

This study investigated the effects of dietary supplementation with *Mansoa alliacea* hydroalcoholic extracts on growth, blood and immune parameters of *Arapaima gigas*. Fish were fed for 30 days with diets enriched with 0, 4, 8, and 12 g kg^{-1} of *M. alliacea* hydroalcoholic extract and subjected to infection with *Aeromonas hydrophila* and handling stress. Fish fed with 8 g kg^{-1} of extract showed significant increase in final weight, specific growth rate and feed efficiency when compared to the other groups. Glucose, triglycerides, total proteins, and globulins increased significantly in fish fed with 8 g kg^{-1} of extract, whereas albumin decreased. The number of thrombocytes increased significantly with the dietary supplementation of 8 and 12 g kg^{-1} of extract. After the challenge with *A. hydrophila* and handling stress, fish fed with 8 g kg^{-1} of extract had significantly higher levels of glucose, globulins, and albumins, and fish fed with 8 and 12 g kg^{-1} of extract showed an increment of respiratory burst. Triglyceride levels dropped significantly in fish fed with 4, 8, and 12 g kg^{-1} of extract, whereas the number of neutrophils increased, and total thrombocytes, leukocytes and lymphocytes were higher in fish fed with 12 g kg^{-1} of extract. Dietary supplementation with *M. alliacea* extract at 8 g kg^{-1} was efficient in improving the growth and innate immunity of *A. gigas*, being potentially useful in fish farming to control the development of *A. hydrophila* infections.

KEYWORDS: diet, immunostimulant; freshwater fish; nutrition; herbal therapy

Extrato de *Mansoa alliacea* melhora o desempenho de crescimento e resposta imunológica de *Arapaima gigas* desafiado com *Aeromonas hydrophila* e estresse de manejo

RESUMO

Investigou-se os efeitos da suplementação com extrato hidroalcólico de *Mansoa alliacea* sobre o crescimento e parâmetros sanguíneos e imunológicos de *Arapaima gigas*. Os peixes foram alimentados por 30 dias com dietas enriquecidas com 0, 4, 8 e 12 g kg^{-1} de extrato hidroalcoólico de *M. alliacea* e submetidos à infecção por *Aeromonas hydrophila* e estresse de manejo. Os peixes alimentados com 8 g kg^{-1} de extrato apresentaram aumento significativo no peso final, taxa de crescimento específico e eficiência alimentar quando comparados aos demais grupos. Glicose, triglicerídeos, proteínas totais e globulinas aumentaram significativamente nos peixes alimentados com 8 g kg^{-1} de extrato, enquanto a albumina diminuiu. O número de trombócitos aumentou significativamente com a suplementação dietética de 8 e 12 g kg^{-1} de extrato. Após infecção com *A. hydrophila* e estresse de manejo, os peixes alimentados com 8 g kg^{-1} de extrato apresentaram níveis significativamente mais altos de glicose, globulinas e albuminas, e os peixes alimentados com 8 e 12 g kg^{-1} de extrato apresentaram incremento de explosão respiratória. Os níveis de triglicerídeos decresceram nos peixes alimentados com 4, 8 e 12 g kg^{-1} de extrato, enquanto o número de neutrófilos aumentou, e o número total de trombócitos, leucócitos e linfócitos foi maior nos peixes alimentados com 12 g kg^{-1} de extrato. A suplementação com a 8 g kg^{-1} de extrato de *M. alliacea* foi eficiente em melhorar o crescimento e a imunidade inata de *A. gigas*, sendo potencialmente útil na piscicultura para controlar o desenvolvimento de infecções por *A. hydrophila*.

PALAVRAS-CHAVE: dieta, imunoestimulante; peixe de água doce; nutrição; fitoterapia

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INTRODUCTION

The current aquaculture practices make fish susceptible to infectious disease outbreaks as a result of high stocking density and low water quality, causing physiological changes with negative impact on fish welfare. As a consequence, farmed fish has low growth performance and is more susceptible to diseases (Adel *et al.* 2015; Shakya 2017). Hence, numerous extracts and essential oils from medicinal plants have been tested for improving the growth performance and innate immune system in different fish species (Sahu et al. 2007; Nay and Austin 2009; Bilen *et al*. 2011; Kanani *et al*. 2014; Adel *et al*. 2015; Akrami *et al*. 2015; Inoue *et al*. 2016; Altunoglu *et al*. 2017; Hoseinifar *et al.* 2017; Moghanlou *et al.* 2018; Bilen *et al*. 2019).

Medicinal plants are rich sources of safe and cheaper bioactive compounds, with anti-stress, growth promotion, appetite stimulation, enhancement of tonicity, and immunostimulant properties (Bilen *et al*. 2011; Akrami *et al*. 2015; Adel *et al*. 2015; Inoue *et al*. 2016; Shakya 2017; Altunoglu *et al*. 2017; Moghanlu *et al*. 2018; Bilen *et al*. 2019; Xu *et al*. 2020), providing resistance to pathogens and decreasing stress related to diseases (Inoue *et al*. 2016; Shakya *et al*. 2017). Simultaneously, the dietary supplementation of herbal immunostimulants minimized the risks of toxicity associated with chemical drugs and therefore it represents one of the most promising methods for controlling diseases in aquaculture fish (Sahu *et al*. 2007; Adel *et al*. 2015; Inoue *et al*. 2016; Altunoglu *et al*. 2017; Bilen *et al*. 2019). Herbal therapy may reduce the treatment costs, and may produce more biodegradable compounds than chemical products when released to environment (Altunoglu *et al*. 2017; Shakya 2017; Moghanlu *et al*. 2018).

Arapaima gigas Schinz, 1822 (Arapaimidae) is an important fish species farmed in the Amazon region, reaching up to 3 m and 200 kg (Lima *et al*. 2017). The welfare of aquacultured *A. gigas* can be strongly compromised by stress caused by handling procedures, which are common during the intensive production cycle (Lima *et al*. 2017; Dias *et al.* 2019). Stress can significantly affect fish survival and growth, increasing the susceptibility to infection by *Aeromonas hydrophila* (Chester, 1901) Stanier, 1943 (Dias *et al*. 2016; Dias *et al*. 2019; Proietti-Júnior *et al*. 2021). This bacterium is responsible for significant economic losses in the aquaculture of *A. gigas* (Proietti-Junior *et al*. 2021).

Mansoa alliacea (Lam.) A.H. Gentry (Bignoniaceae) is a medicinal plant that is commonly known as garlic vine, forest garlic, and *ajo sacha* because of the strong garlic scent exhaled when its leaves, stems, flowers, and fruit are macerated. The plant is native to the Amazon region, but it spread to other parts of the world, and usually occurs in non-flooded areas with shade and low vegetation density (Zoghbi *et al*. 2009; Pires *et al*. 2016; Walag *et al*. 2017). This plant contains flavonoids, terpenes, tannins, naphthoquinones, alkaloids, coumarins, saponins, p-coumaric acid, ferulic acid, lapachol, allicin, alliin allyl sulfides resveratrol, and alliin as major compounds (Zoghbi *et al*. 2009; Pires *et al*. 2016; Walag *et al*. 2017). Leaves, vine bark, and root of *M. alliacea* have been extensively used by indigenous tribes and urban populations in the Amazon (Zoghbi *et al*. 2009). The chemical composition of the organic extracts of *M. alliacea* includes alkanes, alkanols, triterpenoids, flavonoids, lapachol derivatives and the organosulfur compound alliin (Zoghbi *et al*. 2009). Plants are rich sources of bioactive compounds that have anti-stress properties, promote growth, stimulate the appetite, and increase the levels of digestive and pancreatic enzymes in supplemented fish (Shakya *et al*. 2017; Bilen *et al*. 2019; Xu *et al.* 2020; Xu *et al.* 2020). Therefore, fish diets supplemented with this medicinal plant could improve fish performance and immunity for resistance to stress and infectious diseases.

To our knowledge, there is no published study on the use of *M. alliacea* as a dietary supplement for fish. Thus, the aim of this study was to evaluate the effect of the hydroalcoholic extract of *M. alliacea* as a dietary supplement on the growth performance, hematological, biochemical and immunological parameters of *A. gigas* before and after being challenged with *A. hydrophilla* and handling stress.

MATERIAL AND METHODS

This study was carried out in accordance with the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee on Ethics in the Use of Animals of Universidade Federal do Acre (CEUA/UFAC protocol # 08/2014).

Fish acquisition and acclimatization

Fingerlings of *A. gigas* (15.0 ± 3.0 g) were acquired from a commercial fish farm in Rio Branco, Acre state, Brazil, and acclimated in a 1000-L tank with a continuous flow of water and constant aeration in Macapá, Amapá state, Brazil (0°0'49.16"S, 51°5'2.40"W). During acclimation fish were fed *ad libitum* with a commercial *extruded* feed containing 55% of crude protein (Presence®, São Paulo, Brazil), four times a day. Every day the organic matter was removed from the bottom of the tanks and the levels of dissolved oxygen (6.6 ± 0.3 mg L^{-1}), temperature (30.1 ± 0.2 °C), and pH (5.4 ± 0.2) were measured with a multiparameter probe (Horiba, model U52, Kyoto, Japan).

Preparation of the hydroalcoholic extract

Leaves of *M. alliacea* were collected in the city of Porto Grande, Amapá state (Brazil), and a voucher was deposited at the herbarium of Instituto de Pesquisa Científica e Tecnológica do state of Amapá, Macapá, Brazil (voucher # tavares-dias001-HAMAB/IEAP). Samples were crushed and macerated in

a glass crucible for hydro-alcoholic (90% ethylic alcohol, Sinth, Brazil) extraction. The proportion of plant to solvent was of 1:10 (w/v). After extraction, the extract was stored at environmental temperature, for 16 h, and protected from light for its subsequent inclusion in the diets of *A. gigas*.

Preparation of the diets

The diets were prepared using a commercial extruded feed with 45% of crude protein (Presence®, São Paulo, Brazil), by adding 0, 4, 8, and 12 g kg^{-1} of *M. alliacea* extract (Inoue *et al.* 2016). The extract was sprayed on the feed and dried at room temperature for 24 h. The analysis of chemical composition of the basal control diet and diets prepared with different *M. alliacea* extract concentrations was carried out in triplicate, according to the guidelines of the Association of Official Analytical Chemistry (AOAC 1995) (Table 1).

Experimental design

The fingerlings were randomly distributed into twelve tanks of 100 L, constituting four treatments of *M. alliacea* extract concentration (0, 4, 8, and 12 g kg^{-1}) with three repetitions per diet (10 fingerlings per replicate, total N = 30). Control fish were fed the basic commercial feed. During the 30 days of the feeding trial, the fingerlings were fed four times a day (8 am, 11 am, 2 pm, and 5 pm) in the proportion of 6% of body weight.

During the entire experiment, the water flow was kept continuous, and the levels of dissolved oxygen (6.8 ± 0.4 mg L^{-1}), temperature (29.3 ± 0.2 °C), and pH (5.3 ± 0.2) were daily measured using a multiparameter probe (Horiba, model U52, Kyoto, Japan).

Fish growth parameters

At the end of the 30-day feeding trial, all fish were weighed and measured for the following growth parameters:

a) Final biomass (kg m^{-3}) = final average weight x total number of fish;

b) Feed conversion ratio (FCR) = average feed intake (kg)/ average biomass gain (kg m^{-3});

c) Daily feed ingestion = amount of feed (g)/time (30 days);

d) Daily weight gain = weight gain (g)/time (30 days);

e) Weight gain = final weight - initial weight;

f) Specific growth rate (SGR) = (ln final weight - ln initial weight) x 100/(days);

g) Feed efficiency (FE) = 100 x [weight gain (g)/amount of ingested feed (g)];

h) Relative condition factor (Kn), according to the method proposed by Le Cren (1951);

i) Hepatosomatic index (%): [liver weight (g) / body weight (g)] x 100;

j) Viscerosomatic index (%): [viscera weight (g) / body weight (g)] x 100.

Hematological, biochemical and immunological parameters

At the end of the feeding trial, five fish from each replicate (15 fish per dietary treatment) were submitted to blood collection through the puncture of the caudal vessel with a syringe containing sodium heparin (5000 UI, Cristália, SP, Brazil). Blood was divided into two aliquots. The first aliquot was used for the determination of hematocrit through the microhematocrit method, count of total erythrocytes in a Neubauer chamber, and hemoglobin concentration through the cyanmethemoglobin method. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated. Blood smears were made and stained panchromatically with a May Grünwald-Giemsa-Wright combination for the differential counting of leukocytes using the traditional method (Ranzani-Paiva *et al.* 2013). The identification and nomenclature of leukocyte populations followed Tavares-Dias *et al.* (2007). Blood smears were also used for the determination of total leucocyte and thrombocyte counts (Ranzani-Paiva *et al.* 2013).

The respiratory burst activity of leukocytes was determined according to the method described by Biller-Takahashi *et al.* (2013a). Briefly, 100 µL of heparinized blood was added to

Table 1. Proximate composition of the experimental diets containing different concentrations of hydrialcoholic extract of *Mansoa alliacea* administered to *Arapaima gigas* fingerlings.

Parameter	*Mansoa alliacea* extract (g kg^{-1})				P-value
	0	4	8	12	
Crude protein (g kg^{-1})	460.0 ± 2.4[a]	467.0 ± 2.2[a]	447.0 ± 1.7[a]	465.0 ± 1.7[a]	0.898
Dry matter (g kg^{-1})	922.0 ± 0.7[a]	914.0 ± 1.7[a]	914.0 ± 1.0[a]	914.0 ± 1.0[a]	0.670
Ethereal extract (g kg^{-1})	45.0 ± 1.0[a]	45.0 ± 0.6[a]	44.0 ± 0.5[a]	44.0 ± 0.8[a]	0.567
Ash (g kg^{-1})	119.0 ± 1.7[a]	119.0 ± 2.7[a]	105.0 ± 4.7[a]	120.0 ± 1.7[a]	0.990
Phosphorus (g kg^{-1})	9.0 ± 0.4[a]	11.0 ± 1.4[a]	11.0 ± 1.0[a]	12.0 ± 0.9[a]	0.566
Calcium (g kg^{-1})	0.9 ± 0.4[a]	0.9 ± 0.6[a]	2.2 ± 0.7[b]	3.9 ± 0.3[c]	0.010

Mean values ± standard error of the mean. Three replicates per dietary treatments (N = 3). Composition of micronutrients and energy of the basal diet: Energy: 3250 kcal kg^{-1}; Vitamin E: 400 mg kg^{-1}; Calcium = 2-3%; Vitamin C: 1500 mg kg^{-1}; Crude fiber: 36 g kg^{-1}. Mean values followed by different letters, on the same line, indicate significant pairwise difference between diets by the Tukey test ($p < 0.05$).

100 µL of a 0.2% nitro blue tetrazolium solution (Sigma, St. Louis, MO, USA) and the final suspension was homogenized and incubated for 30 min at 25 °C. After incubation and a second homogenization, 50 µL of the obtained solution was added to 1 mL of N, N-dimethyl formamide (Sigma, St. Louis, MO, USA). This further solution was homogenized and centrifuged at 3,000 x g for 5 min. The optical density of the supernatant was determined on a spectrophotometer (Biospectro SP-220, Curitiba, Brazil) at 540 nm.

The second aliquot of blood was centrifuged at 1500 x g for 5 min (Mod. 5424, Hamburg, Germany) for obtaining the plasma and determining the concentration of glucose, total proteins, albumin, cholesterol, and triglycerides using colorimetric kits (Biotécnica, MG, Brazil) and reading the absorbance by a spectrophotometer (Biospectro SP-220, Curitiba, Brazil). The globulin concentration was determined from the difference between total protein and albumin levels.

Challenge with *Aeromonas hydrophila* and handling stress

After thirty days of feeding, the remaining five fish from each replicate (15 fish per dietary treatment) were challenged with *A. hydrophila* (ATCC 7966). Fish were intraperitoneally inoculated with 50% of lethal dose ($LD_{50\text{-}96h}$) of 1.8 x 10^8 UFC mL^{-1} (Dias et al. 2016). Survival and clinical signs of disease in fish were observed for seven days, then fish were subjected to handling stress. The fish were individually captured and maintained out of the water for 60 seconds (Barton and Zitzow 1995; Davis and Schreck 1997). After 6 hours post-stress, blood was collected from each fish as previously mentioned and divided into two aliquots for the determination of hematocrit, hemoglobin concentration, number of total erythrocytes, thrombocytes, leukocytes, leukocyte respiratory activity, levels of glucose, total proteins, albumin, cholesterol, triglycerides, and globulins, as described above.

Statistical analyses

All data were evaluated for the assumptions of normality and homoscedasticity using Shapiro-Wilk and Bartlett tests, respectively. The response variables that displayed a normal distribution were compared among the four experimental diets (N = 3 per diet) with analysis of variance (ANOVA-one-way) followed by the post-hoc Tukey test for pairwise comparison of means. Data that did not present a normal distribution were analyzed using the Kruskal-Wallis test, followed by the Dunn test for the comparison among medians (Zar 2010). A significance level of 5% ($p < 0.05$) was adopted for all analyses.

RESULTS

Fish fed the diet enriched with 8 g kg^{-1} of *M. alliacea* extract showed a significant increase in the final weight, final biomass, daily weight gain, weight gain, specific growth rate, and feed efficiency compared to fish fed the other diets (Table 2). Plasma glucose, triglycerides, total proteins, and globulins significantly increased in fish fed the 8 g kg^{-1} diet, while albumin levels significantly decreased. Total thrombocyte number was significantly higher in fish fed the 8 and 12 g kg^{-1}

Table 2. Growth performance parameters of *Arapaima gigas* fed with three experimental diets supplemented with different concentrations of hydrialcoholic extract of *Mansoa alliacea* (or a control diet) for 30 days.

Parameters	*Mansoa alliacea* extract (g kg^{-1})				P-value
	0	4	8	12	
Initial length (cm)	26.2 ± 1.1[a]	25.6 ± 1.2[a]	24.8 ± 5.1[a]	25.1 ± 1.2[a]	0.871
Initial weight (g)	126.4 ± 4.1[a]	124.6 ± 4.3[a]	129.3 ± 22.6[a]	123.1 ± 4.6[a]	0.890
Final length (cm)	37.7 ± 1.6[a]	37.4 ± 1.9[a]	38.9 ± 3.8[a]	37.0 ± 1.6[a]	0.567
Final weight (g)	302.9 ± 8.8[a]	288.9 ± 13.2[a]	358.9 ± 79.0[b]	280.5 ± 8.4[a]	0.005
Final biomass (kg m^{-3})	30.3 ± 2.0[a]	28.9 ± 3.1[a]	37.7 ± 1.0[b]	27.4 ± 1.6[a]	0.001
Daily weight gain (g)	5.9 ± 1.9[a]	5.5 ± 2.5[a]	7.5 ± 2.9[b]	5.6 ± 1.7[a]	0.001
Weight gain (g)	176.5 ± 9.6[a]	164.3 ± 74.0[a]	227.6 ± 86.4[b]	167.4 ± 10.6[a]	0.041
Daily feed intake (g)	70.9 ± 1.6[a]	71.1 ± 0.7[a]	72.0 ± 1.0[a]	71.8 ± 1.0[a]	0.881
FCR	1.6 ± 0.4[a]	1.7 ± 1.14[a]	1.6 ± 0.4[a]	1.6 ± 0.7[a]	0.991
SGR	2.8 ± 1.3[a]	2.7 ± 1.0[a]	3.5 ± 1.1[b]	2.8 ± 1.0[a]	0.041
Feed efficiency (%)	66.6 ± 17.5[a]	61.7 ± 3.5[a]	94.6 ± 3.8[b]	61.1 ± 1.5[a]	0.002
Condition factor	1.00 ± 0.2[a]	1.00 ± 0.2[a]	1.00 ± 0.2[a]	1.00 ± 0.5[a]	0.786
HSI (%)	2.0 ± 0.4[a]	2.4 ± 0.4[a]	2.2 ± 0.7[a]	2.2 ± 0.7[a]	0.689
VSI (%)	9.7 ± 1.[7a]	9.8 ± 1.7[a]	9.2 ± 1.2[a]	10.4 ± 1.2[a]	0.760

Mean values ± standard error of the mean. Three replicates per dietary treatments (N = 30). HSI: hepatosomatic index; VSI: viscerosomatic index; FCR: feed conversion ratio; SGR: specific growth rate. Mean values followed by different letters, on the same line, indicate significant pairwise differences between dietary treatments by the Tukey test ($p < 0.05$).

diets when compared to the control group. Plasma cholesterol, hematocrit, hemoglobin, erythrocytes, MCV, MCHC, leukocyte number and respiratory burst of leukocytes were not affected by the dietary treatments (Table 3).

After the *A. hydrophila* challenge and handling stress, mortality occurred only in control fish and fish fed the 4 g kg^{-1} diet. Fish fed the 8-g kg^{-1} diet showed a significant increase in levels of plasma globulin and glucose, while triglyceride levels decreased significantly in the fish fed all supplemented diets. Fish fed the 8 and 12-g kg^{-1} diets displayed a significant raise in leukocyte respiratory burst compared to the other treatments. The number of neutrophils significantly increased in all treatments compared to the control. The total number of thrombocytes, total leukocytes and lymphocytes also increased in fish fed the 12 g kg^{-1} diet relative to the other treatments (Table 4).

DISCUSSION

As already mentioned, *M. alliacea* contains flavonoids, terpenes, tannins, naphthoquinones, alkaloids, coumarins, saponins, p-coumaric acid, ferulic acid, lapachol, alliin allyl sulfides resveratrol, allicin, and alliin as major compounds (Zoghbi *et al*. 2009; Pires *et al.* 2016; Walag *et al.* 2017). Allicin is a compound that induces an increase in feed intake, while terpenes and coumarins are growth regulators in animals (Shakya *et al*. 2017; Bilen *et al*. 2019; Xu *et al*. 2020). The antimicrobial activity attributed to several plants may be related to the presence of alkaloids, tannins, and flavonoids in their composition (Pires *et al.* 2016). To the best of our knowledge, the current study is the first to test *M. alliacea* as a growth promoter and immunostimulant in fish. The dietary supplementation with 8 g kg^{-1} of *M. alliacea* hydroalcoholic extract improved the final weight, final biomass, daily weight gain, specific growth rate, and feed efficiency of *A. gigas* fingerlings. Similarly, the inclusion in the diet of 0.5-10 g kg^{-1} of *Allium sativum* Linnaeus improved the weight gain, specific growth rate, feed conversion and protein efficiency rate of *Oncorhynchus mykiss* Walbaum, 1792 (Nay and Austin 2009). The weight and specific growth rates were also improved after feeding *Huso huso* Linnaeus, 1758 with diets containing 5 and 10 g kg^{-1} of *Allium cepa* Linnaeus (Akrami *et al*. 2015), and *Salmo caspius* Kessler, 1877 with diets containing the essential oil of *Mentha piperita* Linnaes (Adel *et al.* 2015). In contrast, the dietary supplementation with *Nigella sativa* Linnaeus reduced the weight gain and specific growth rate of *O. mykiss* (Altunoglu *et al*. 2017), whereas diets enriched with *Cotinus coggygria* Scop (Bilen *et al.* 2011) and *Zingiber officinale* Roscoe (Kanani *et al*. 2014) did not influence its growth. Several authors reported that bioactive compounds of such medicinal plants stimulate the digestive process of fish, thus incrementing the feed efficiency and growth (Bilen

Table 3. Hematological and immunological parameters of *Arapaima gigas* fed with the experimental diets supplemented with different levels of *Mansoa alliacea* hydroalcoholic extract for 30 days.

Parameter	*Mansoa alliacea* extract (g kg^{-1})				P-value
	0	4	8	12	
Glucose (mg dL^{-1})	32.1 ± 4.3^a	37.3 ± 15.7^a	74.2 ± 5.3^b	33.6 ± 4.1^a	0.021
Total cholesterol (mg dL^{-1})	109.7 ± 5.1^a	129.4 ± 5.6^a	144.8 ± 6.7^a	144.9 ± 4.8^a	0.567
Triglycerides (mg dL^{-1})	152.5 ± 6.9^a	144.8 ± 5.3^a	236.4 ± 8.7^b	128.5 ± 7.1^a	0.890
Total protein (g dL^{-1})	2.8 ± 0.7^a	2.9 ± 0.7^a	5.2 ± 2.1^b	3.5 ± 1.0^a	0.001
Albumin (g dL^{-1})	1.2 ± 0.6^a	1.0 ± 0.7^a	0.6 ± 0.7^b	1.3 ± 0.6^a	0.001
Globulin (g dL^{-1})	1.6 ± 0.7^a	1.9 ± 0.7^a	4.7 ± 2.1^b	2.2 ± 1.0^a	0.001
Hematocrit (%)	28.8 ± 1.3^a	28.5 ± 1.3^a	30.1 ± 1.3^a	27.6 ± 1.4^a	0.821
Hemoglobin (g/dL)	4.4 ± 0.7^a	4.7 ± 0.8^a	5.0 ± 0.6^a	4.6 ± 0.8^a	0.999
Erythrocytes (x 10^6 μL^{-1})	2.2 ± 0.7^a	2.1 ± 0.8^a	2.4 ± 1.0^a	2.2 ± 0.8^a	0.999
MCV (fL^{-1})	143.2 ± 6.1^a	142.9 ± 5.8^a	155.4 ± 8.9^a	129.3 ± 5.0^a	0.565
MCHC (g dL^{-1})	15.3 ± 21.6	16.5 ± 1.5^a	16.7 ± 1.3^a	16.9 ± 1.8^a	0.667
Respiratory burst (OD)	0.19 ± 0.1^a	0.23 ± 0.3^a	0.21 ± 0.3^a	0.17 ± 0.2^a	0.876
Thrombocytes (x 10^3 μL^{-1})	29.9 ±10.8^a	44.4 ± 4.2^{a,b}	49.1 ± 5.1^b	52.6 ± 4.0^b	0.001
Leukocytes (x 10^3 μL^{-1})	237.9 ± 77.9^a	254.9 ± 8.8^a	288.3 ± 9.7^a	276.8 ± 8.5^a	0.997
Lymphocytes (x 10^3 μL^{-1})	114.3 ± 3.3^a	124.7 ± 7.0^a	164.4 ± 9.1^a	155.9 ± 6.2^a	0.997
Monocytes (x 10^3 μL^{-1})	8.1 ± 2.3^a	6.1 ± 2.0^a	11.1 ± 3.1^a	12.4 ± 2.9^a	0.990
Neutrophils (x 10^3 μL^{-1})	112.6 ± 7.3^a	119.5 ± 6.2^a	109.4 ± 6.6^a	102.1 ± 5.4^a	0.812
Eosinophils (x 10^3 μL^{-1})	2.9 ± 1.6^a	4.5 ± 1.7^a	3.3 ± 1.5^a	6.4 ± 2.7^a	0.567

Mean values ± standard error of the mean. Three replicates per dietary treatments (N = 15). MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration. Mean values followed by different letters, on the same line, indicate significant pairwise differences among dietary treatments by the Dunn test ($p < 0.05$).

Table 4. Hematological, biochemical and immunological parameters of *Arapaima gigas* fed with experimental diets containing different concentratios of *Mansoa alliacea* hydroalcoholic extract after being challenged with *Aeromonas hydrophila* and handling stress.

Parameter	*Mansoa alliacea* extract (g kg^{-1})				P-value
	0	4	8	12	
Mortality (%)	40	10	0	0	-
IHS (%)	1.8 ± 0.7^a	1.7 ± 0.6^a	1.7 ± 0.7^a	1.4 ± 0.7^a	0.899
SSI (%)	0.2 ± 0.3^a	0.2 ± 0.3^a	0.2 ± 0.3^a	0.1 ± 0.3^a	0.778
Glucose (mg dL^{-1})	38.3 ± 3.0^a	49.4 ± 4.4^a	113.7 ± 5.0^b	78.4 ± 3.7^a	0.039
Total protein (g dL^{-1})	3.5 ± 0.6^a	3.3 ± 1.0^a	4.3 ± 1.0^a	3.1 ± 1.0^a	0.567
Cholesterol (mg dL^{-1})	155.4 ± 6.0^a	167.4 ± 5.3^a	199.1 ± 76.5^a	156.2 ± 5.4^a	0.778
Triglycerides (mg dL^{-1})	135.0 ± 7.8^a	66.7 ± 5.4^b	64.8 ± 8.7^b	67.9 ± 4.3^b	0.001
Albumin (g dL^{-1})	± 0.5^a	0.5 ± 0.4^a	0.5 ± 0.6^a	1.0 ± 0.2^a	0.559
Globulin (g dL^{-1})	2.5 ± 0.7^a	2.8 ± 0.8^a	3.8 ± 0.8^b	2.1 ± 1.0^a	0.030
Hematocrit (%)	33.6 ± 1.5^a	33.7 ± 1.6^a	29.3 ± 1.7^a	29.8 ± 1.5^a	0.336
Hemoglobin (g dL^{-1})	5.7 ± 1.2^a	5.9 ± 1.2^a	4.7 ±1.1^a	5.7 ± 1.0^a	0.289
Erythrocytes (x 10^6 µL^{-1})	1.4 ± 0.7^a	1.6 ± 0.6^a	1.6 ± 0.7^a	2.3 ± 0.5^a	0.789
MCV (fL^{-1})	270.3 ± 8.9^a	218.2 ± 8.2^a	196.7 ± 8.6^a	133.6 ± 4.8^a	0.234
MCHC (g dL^{-1})	16.8 ± 1.7^a	17.7 ± 2.0^a	16.9 ± 1.9^a	19.1 ± 1.8^a	0.890
Respiratory burst	0.15 ± 0.4^a	0.15 ± 0.3^a	0.29 ± 0.5^b	0.28 ± 0.2^b	0.010
Thrombocytes (x 10^3 µL^{-1})	26.2 ± 9.9^a	31.9 ± 3.3^a	36.6 ± 3.5^a	63.7 ± 3.8^b	0.041
Leukocytes (x 10^3 µL^{-1})	162.5 ± 3.1^a	197.0 ± 6.8^a	192.4 ± 8.2^a	290.8 ± 39.3^b	0.045
Lymphocytes (x 10^3 µL^{-1})	106.3 ± 5.8^a	129.4 ± 5.7^a	120.2 ± 6.8^a	186.0 ± 6.3^b	0.022
Monocytes (x 10^3 µL^{-1})	6.6 ± 1.8^a	8.3 ± 2.1^a	9.0 ± 1.9^a	15.3 ± 2.5^a	0.443
Neutrophils (x 10^3 µL^{-1})	47.6 ± 4.3^a	57.0 ± 4.5^b	59.5 ± 5.3^b	85.2 ± 6.1^b	0.001
Eosinophils (x 10^3 µL^{-1})	2.1 ± 1.3^a	2.3 ± 1.6^a	3.6 ± 1.7^a	4.2 ± 2.1^a	0.778

Mean values ± standard error of the mean. Three replicates per dietary treatments (N = 15). MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; HSI: hepatosomatic index; SSI: splenic somatic index. Mean values followed by different letters, on the same line, indicate significant pairwise differences among dietary treatments by the Dunn test (p< 0.05).

et al. 2011; Kanani *et al.* 2014; Adel *et al.* 2015; Altunoglu *et al.* 2017).

Aquacultured fish represent a significant resource in the world to meet the needs of food of a human population in fast growth. Therefore, the health condition of farmed fish must be carefully monitored to limit the development of diseases and stress, which negatively impact the productivity of this sector (Altunoglu *et al.* 2017). Physiological and immunological parameters may be important indicators to monitor the welfare of farmed fish in response to nutrition, diseases and stress (Nay and Austin 2009; Razani-Paiva *et al.* 2013; Inoue *et al.* 2016). In particular, high levels of total proteins and globulins have been associated with a strong response of the innate immune system of fish (Akrami *et al.* 2015). The dietary supplementation with 8 g kg^{-1} of *M. alliacea* extract increased the plasma levels of glucose, triglycerides, total proteins and globulins, while reducing the albumin levels in *A. gigas* fingerlings. Therefore, this plant seems to have hyperlipidemic and hyperglycemic effects, which must be better investigated. In addition, the diets enriched with 8 and 12 g kg^{-1} of extract raised the number of total thrombocytes. The inclusion of *A. cepa* in the diet reduced the levels of glucose, total proteins, triglycerides, cholesterol, albumin, and globulins, but increased the hematocrit and the number of erythrocytes and leukocytes in *H. huso* (Akrami *et al.* 2015). The parameters of erythrocytes and leukocytes increased in *O. mykiss* fed diets supplemented with *A. sativum* according to the concentrations used, except for hemoglobin and the number of thrombocytes (Nay and Austin 2009). In contrast, the dietary supplementation with *A. sativum* did not significantly influence the levels of glucose, total proteins, and erythrocyte parameters of *Colossoma macropomum* Cuvier, 1818, yet it reduced the number of total leukocytes, lymphocytes and neutrophils (Inoue *et al.* 2016).

Arapaima gigas often suffers from bacteriosis problems (Dias *et al.* 2016; Proietti-Junior *et al.* 2021) caused by stress inherent to the handling, thus requiring prophylactic measures to allow its intensive production. *Mansoa alliacea* has been widely used in the treatment of several illnesses in humans due to the presence of coumarins and phenolic compounds, which aid in the release of histamine and explain anti-inflammatory and antimicrobial activity, and immunostimulant properties (Zoghbi *et al.* 2009; Pires *et al.* 2016). In *A. gigas* fed diet with 8 g kg^{-1} of extract and challenged with *A. hydrophila*

and handling stress, a significant increase in the levels of glucose, globulins, leukocyte respiratory burst, and number of neutrophils was observed. In general, serum or plasma glucose, cortisol and lysozyme activity are considered as indicators of stress in fish (Urbinati *et al*. 2020). In the present study, fish showed a significant increase of plasma glucose both after feeding with supplemented diets and after challenge with *A. hydrophila* and handling. Therefore, the results demonstrated that *M. alliacea* extract at the tested concentrations did not explain anti-stress properties in *A. gigas*. In addition, the dietary supplementation with 12 g kg^{-1} of extract raised the leukocyte respiratory burst and the numbers of thrombocytes, total leukocytes, and lymphocytes and neutrophils. The simultaneous increase of leukocyte respiratory burst and neutrophil number could be considered an indicator of enhancement of the defense mechanisms against bacterial infections due to the rise in the phagocyte activity (Bilen *et al.* 2011; Biller-Takahashi *et al.* 2013a), and also the increment in the globulin levels may indicate an improvement of immune response against *A. hydrophila*. No mortality was observed in fish fed with diets containing 8 and 12 g kg^{-1} of *M. alliacea* extract that were inoculated with a subletal concentration of *A. hydrophila*, because a potential immune stimulatory effect was expected to give protection against this bacterium.

CONCLUSIONS

This study showed the potential use of *Mansoa alliacea* hydroalcoholic extract in aquaculture in promoting growth performance and innate immunity in *Arapaima gigas* fingerlings. In particular, the dietary administration of 8 g kg^{-1} of *M. alliacea* extract in *A. gigas* fingerlings for a longer period than 30 days could be evaluated for obtaining improved growth and immune response to infectious diseases in intensive farming.

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